STUDIES WITH CATION SPECIFIC IONOPHORES SHOW THAT WITHIN THE INTACT CHLOROPLAST Mg^{++} ACTS AS THE MAIN EXCHANGE CATION FOR H^+ PUMPING

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1. Introduction

It has recently been reported by Krause [1] and from our laboratory [2,3] that the slow chlorophyll fluorescence quenching observed with chloroplasts isolated so as to retain their outer membranes is due to an energy dependent net movement of cations from the granal to the stromal compartment. Experiments have shown that the high fluorescing state, which exists before illuminating a dark pretreated chloroplast suspension, is an indicator of the presence of inorganic cations, other than protons, within the grana. On the initiation of light induced electron transport and its associated proton pumping these cations are driven from the thylakoid interiors in exchange for the incoming H^+ and this brings about the quenching of chlorophyll fluorescence. As would be expected the collapse of the H^+ gradient by darkness, by blocking electron flow with DCMU or by using appropriate uncoupling conditions reverses the quenching process. If the intact chloroplasts are subjected to osmotic shock to remove their outer membranes there is sufficient cation leakage so that the fluorescence is lowered to the quenched level and the light induced slow induction kinetics are lost. However, addition of inorganic cations to these osmotically shocked chloroplasts re-establishes the high fluorescing state and the light induced quenching phenomenon. It was found that Mg^{++} or Ca^{++} at about 3 to 5 mM was sufficient to regenerate the fluorescence properties although for K^+ or Na^+ it was necessary to add in excess of 100 mM.

Naturally the question arises as to the nature of the cations which bring about the fluorescence changes within the intact organelle. Recently we used the ionophore A23187 which indicated that Ca^{++} or Mg^{++} may be involved but our experiments did not rule out the involvement of monovalent cation [2]. In this paper we have extended this work to include other cation specific ionophores and moreover we have conducted our experiments with intact chloroplasts under carefully controlled ionic conditions. Our experiments strongly indicate that only Mg^{++} movement controls fluorescence levels in vivo and that this ion probably acts as the main exchange cation for the proton pump.

Abbreviations: CCCP: carbonylcyanide *M*-chlorophenylhydrazone; DCMU: 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; PGA: phosphoglycerate; HEPES: *N*-2-hydroxyethylpiperazine-*N*′-2-ethenasulphonic acid; Tris: Tris (hydroxymethyl)-methylamine.

2. Materials and methods

Intact chloroplasts were isolated from spinach leaves by the method of Stokes and Walker [4]. In our case however, the final suspension medium consisted of 0.33 M sorbitol and 10 mM HEPES adjusted to pH 7.6 with Tris base. The preparations contained about 65% intact chloroplasts as determined by the ferricyanide method [5]. For chlorophyll fluorescence measurements the chloroplast suspension was illuminated in a 1 cm^2 cuvette with blue light at 70 kergs cm^{-2} sec^{-1} transmitted by a filter combination consisting of a Balzer Calflex C, 4 mm Schott BG 18. The emission was detected at right angles to the exciting light by an EMI 9558 photomultiplier screened by a Balzer 695

interference filter and 6 mm of Schott RG665 cut-off filters. All measurements were made at room temperature.

Ionophore A23187 was obtained from Eli Lilly, Indianapolis (Lot No. 361-066-275) as was nigericin (Lot No. 189-380B-171-A) and valinomycin (Lot No. 488-833B-118-2). Beauvaricin was a gift from Dr R. Prince and originated from Dr R. Roeska, Indiana University [6] while the CCCP was purchased from Calbiochem.

A23187 acts as neutral exchanger of protons with divalent cations showing no specificity difference between Ca^{++} and Mg^{++}. Its properties have recently been reviewed by Reed and Lardy [7] and it has been shown to act as an uncoupler of photosynthetic electron flow in the presence of Mg^{++} or Ca^{++} but not K^+ or Na^+ [2]. The actions of nigericin, valinomycin and CCCP are well documented [8,9]. Nigericin facilitates the neutral exchange of K^+/H^+ across the thylakoids while low concentrations of CCCP and valinomycin increase the conductance of these membranes to H^+ and K^+ respectively. Beauvaricin has been reported to transport calcium but not magnesium electrophoretically across liposome membranes and also to show some specificity for monovalent cations [10].

3. Results

Typical time courses for slow chlorophyll fluorescence quenching are shown in fig. 1. These intact chloroplasts were suspended in a medium free of inorganic cations. Under these conditions we have used the above mentioned ionophores to investigate the nature of the intrachloroplast cations involved in the quenching process. As can be seen nigericin did not reverse the quenching and nor did low concentrations of valinomycin in the presence of CCCP. However,

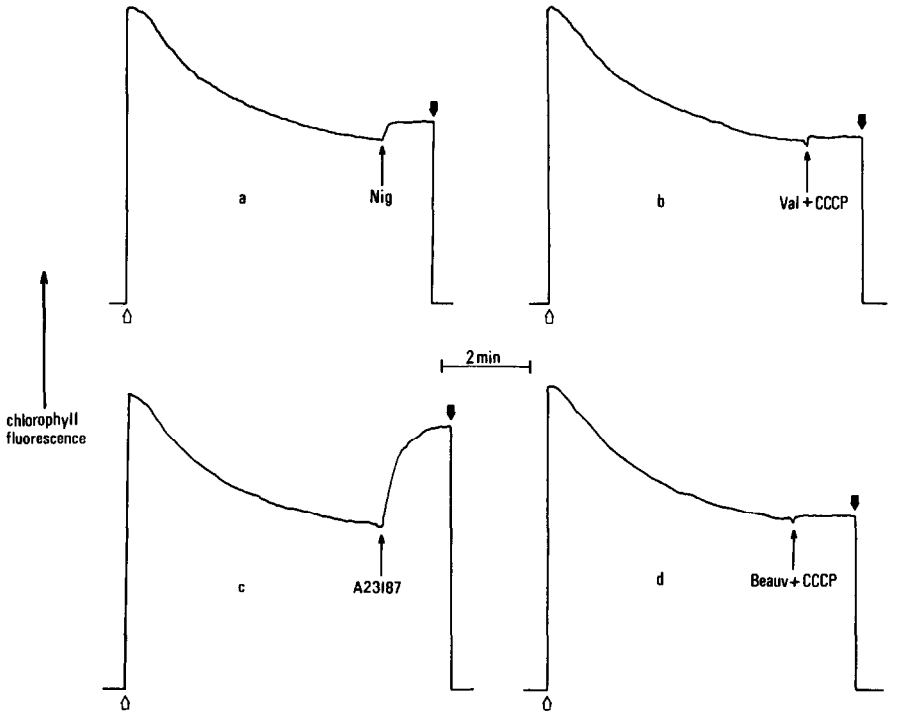

Fig. 1. Effect of (a) 0.05 μM nigericin, (b) 10^{-7} M valinomycin plus 2×10^{-7} M CCCP (c) 1 μg/ml Ionophore A23187 and (d) 0.3 μg/ml Beauvaricin plus 2×10^{-7} M CCCP on light induced quenched state observed with whole chloroplasts suspended in 0.33 M sorbitol, 1 mM PGA and 10 mM HEPES adjusted to pH 7.6 with Tris base. The chlorophyll concentration was 30 μg/ml. Open and closed arrows indicate light on and off.

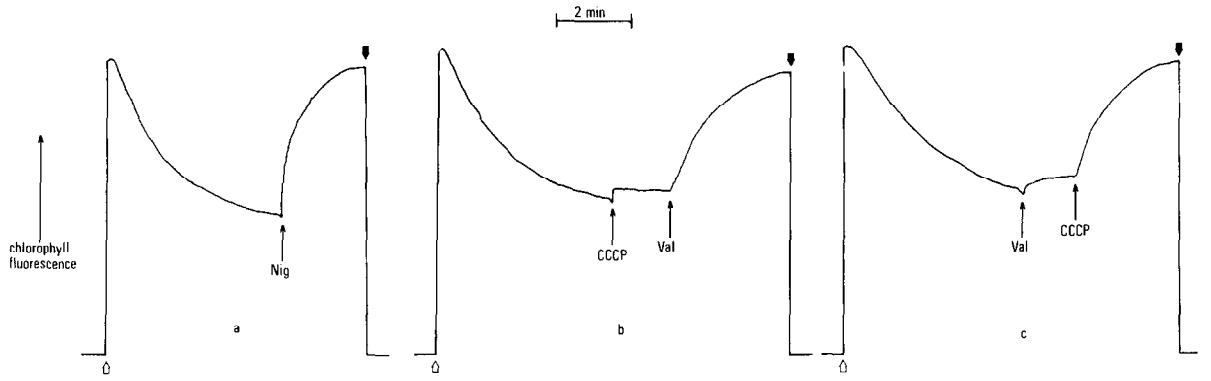

Fig. 2. Effect of (a) 0.05 μM nigericin and (b) and (c) 10^{-7} M valinomycin and 2 × 10^{-7} M CCCP on fluorescence quenching. Exactly the same conditions as fig.1 except 2 mM KCl had been added to the suspending medium.

these same treatments did reverse the quenching if 2 mM K^+ was added to the suspending medium as shown in fig. 2. In the case of valinomycin and CCCP the two compounds act synergistically at these concentrations. Apparently there is insufficient free K^+ within the intact chloroplast to enable either nigericin or valinomycin plus CCCP to dissipate the H^+ gradient and allow the high fluorescing state to be re-established. In the presence of a low level of K^+ in the external medium these treatments must facilitate the transport of this cation across the outer membranes and enable uncoupling to occur at the thylakoids.

On the other hand in the absence of added inorganic cations 1 μg/ml A23187 reversed the quenching (see fig. 1). A23187 facilitates the exchange of H^+ with either Mg^{++} or Ca^+ and this result indicates that either or both these cations are available in the stroma for exchange with H^+ across the thylakoids.

The finding that probably divalent and not monovalent cations control the fluorescence changes in intact chloroplasts led us to use the antibiotic Beauvaricin. As pointed out above it has been reported that this compound transports Ca^{++} but not Mg^{++} electrophoretically across lipid membranes. In fact as fig. 1 shows we found that in the presence of low concentrations of CCCP, added to facilitate the counter transport of H^+, Beauvaricin did not reverse the quenching with intact chloroplasts. However, as shown in fig. 3 this compound did relieve the quenching if 1 mM Ca^{++} was added to the external medium.

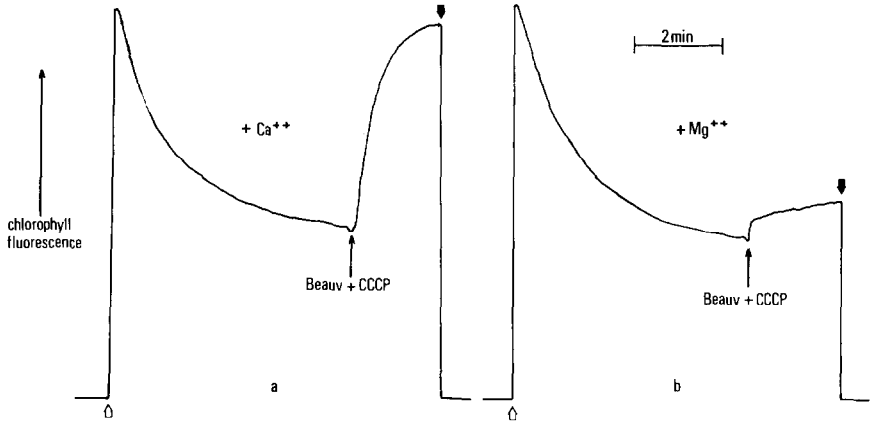

Fig. 3. Experiment (d) of fig.1 conducted after addition of (a) 1 mM $CaCl_2$ and (b) 1 mM $MgCl_2$.

It should also be mentioned that although these experiments were conducted in the presence of CCCP, Beauvaricin at higher concentrations (above 1 μg/ml) reversed the quenching in a Ca^{++} dependent reaction in the absence of CCCP. It seems that this compound must at higher concentrations allow a Ca^{+}–H^{+} exchange to occur.

In order to support the finding that Mg^{++} acts in vivo as the main counterion for H^{+} pumping we have looked at electron transport to PGA under the same conditions as those used for the fluorescence studies. Since the reduction of PGA requires ATP then the effect of uncoupling is to stop net electron flow monitored as oxygen evolution. We found that under the same conditions as those used for the experiments shown in fig. 1, nigericin and valinomycin plus CCCP only totally inhibited electron flow after the addition of 2 mM K^{+} to the cation free medium. Similarly Beauvaricin + CCCP needed Ca^{++} and not Mg^{++} in the medium to inhibit electron flow. On the other hand, in agreement with the fluorescence measurement, A23187 inhibited oxygen evolution without addition of Ca^{++} or Mg^{++} to the cation free medium. The Ca^{++} versus Mg^{++} specificity of Beauvaricin was also clearly seen in a study of its ability to uncouple electron flow to ferricyanide in osmotically shocked chloroplasts.

4. Discussion

For sometime now there have been hints that within the intact chloroplasts Mg^{++} may play an important role in net ionic movement between the grana and stroma [11]. Such movements have been implicated with mechanisms which may control the distribution of light energy to the two photosystems [12] and with the regulation of CO_2 fixation [13]. The early work of Dilley and Vernon [14] and Nobel and Packer [15] and the more recent work of Hind et al. [16] indicated that Mg^{++} may act as a counterion for the light induced uptake of H^{+} across the thylakoids. The experiments reported in this paper give strong support to the idea that light induced Mg^{++}/H^{+} exchange occurs within the intact chloroplast. Perhaps, however, the most surprising feature of our experiments is the apparent lack of involvement of monovalent cations, particularly K^{+}, in the exchange process. Naturally we considered the possibility that by resuspending our chloroplasts in the cation free medium there could have been a sufficient K^{+} leakage so that the levels within the organelle were too low to act as an exchanger with protons and inhibit the uncoupling action of nigericin. However, flame photometry analyses did not support this. We found that under the experimental conditions described in fig. 1 legend the chloroplasts contained 1.7 μmol K^{+}/mg chlorophyll which is consistent with the values given by Nobel [17]. Since this level is in excess of that required to balance the light induced H^{+} movements, which could be in the region of 0.6 μmol H^{+}/mg chlorophyll [16], it must be assumed that for some reason the K^{+} within the chloroplast is not available for exchange with H^{+} either in the presence or absence of nigericin. Hind et al. came to a similar conclusion from their careful studies with broken chloroplasts [16].

Thus our results suggest that within the intact chloroplast Mg^{++} acts as the cation exchanger for the H^{+} pump so that in the light the stromal level of this divalent cation rises as a result of its displacement from the granal compartment. Apparently it is this net movement of Mg^{++} which controls the level of chlorophyll fluorescence. Whether Mg^{++} acts as the only counter ion for the proton pump is uncertain. Hind et al. [16] have suggested that Cl^{-}, as well as Mg^{++}, may be involved and the actual mechanisms of two exchanges could be different. The lack of involvement of K^{+} is strange bearing in mind that this is the major cation in the chloroplast [17] and this observation needs further study.

5. Acknowledgements

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